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# A glimpse into the sexual biology of the "zygiellid" spider genus *Leviellus*

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**Abstract.** We investigated the mating biology of the previously unstudied central European spider *Leviellus thorelli* (Ausserer 1871) by staging laboratory mating trials using males and females of varying mating histories. Our aim was to seek common themes in sexual behaviors of the sexually size-monomorphic "zygiellid" spiders with their putatively close relatives, araneids and nephilids, which are relatively well studied with respect to sexual biology. We found *L. thorelli* mating biology to more closely resemble that of sexually size-monomorphic araneids than that of dimorphic nephilids. Unlike in nephilids with sexually conflicted adaptations, we found no evidence for genital damage or plugging in *Leviellus* Wunderlich 2004, although we found rare cases of half-eunuchs. We suggest that the mating system of *L. thorelli* spiders is determined by short female sexual attractiveness, reduced receptivity after mating and/or intensive mate guarding.

**Keywords:** Mating system, genital plugging, mate guarding, sexual-size dimorphism, eunuchs

Sexual conflict theory concerns the idea that males and females may have different goals in reproduction (Watson 1991; Chapman et al. 2003; Arnqvist & Rowe 2005). As a consequence of intersexual conflict, various morphological, physiological and behavioral adaptations have evolved, such as complex genitalia, multiple sperm storage organs, toxicity of seminal fluids, sexual cannibalism, and mate guarding (Parker 1984; Austad 1984; Chapman et al. 1995; Kuntner et al. 2009a; Uhl et al. 2010). These adaptations along with other demographic and ecological factors shape the mating system of a species.

Among invertebrates, spiders represent an especially suitable clade for sexual selection research (Eberhard 2004). In spiders, the prevailing mating strategy may largely be determined by two morphological constraints: genital morphology and delayed female maturation. First, spiders are classified into entelegyne and haplogyne species (Austad 1984; Uhl 2000; Uhl et al. 2010; Kuntner et al. 2009a). Haplogyne species possess a single insemination duct connected to spermathecae exhibiting last-male sperm priority (Austad 1984; Uhl 2000; Uhl et al. 2010). Alternatively, the entelegyne spiders have separate insemination and fertilization ducts connected to spermathecae and overwhelmingly exhibit first-male sperm priority (Austad 1984; Uhl 2000; Uhl et al. 2010). As a consequence, males of many entelegyne species have evolved mechanisms to avoid or reduce sperm competition with rival males by pre- or post-copulatory mate guarding and by the production of mating plugs (reviewed in Uhl et al. 2010). Although these plugs are thought to largely prevent or delay subsequent mating, they are not universally effective even in closely related species, as studies on nephilid spiders have shown [contrast e.g., *Nephila pilipes* (Fabricius 1793), *Nephilengys malabarensis* (Walckenaer 1841) and *Herennia multipuncta* (Doleschall 1859)]: Fromhage et al. 2007; Schneider et al. 2008; Kuntner et al. 2009b; Kralj-Fišer et al. 2011). Besides mechanical plugging of stored sperm and mate guarding, males employ other mechanisms to reduce sperm competition. Such an example is chemical manipulation, where products of male genitalia that are transferred during copulation may induce female resistance for further matings or earlier oviposition (Eberhard 1997).

Further behavioral and physiological adaptations also shape the mating system of a given species. For example, in highly dimorphic species that produce mating plugs, the small males are often cannibalized after copulation (Nessler et al. 2009), either due to intersexual conflict (Arnqvist & Rowe 2005; Fromhage & Schneider 2005) or male sacrifice, which may have a selective advantage in increasing paternity (Elgar & Nash 1988; Andrade 1996; Elgar et al. 2000; Schneider et al. 2000) leading to monogynous mating systems. In some species, males are physiologically limited to one mating (Downes 1978; Michalik et al. 2010) or females are receptive to only one mate (Alcock & Buchmann 1985).

Finally, female maturation in extremely sexually size dimorphic species is usually considerably delayed (Higgins 2000; Kuntner & Coddington 2009; Kuntner et al. 2009b). Along with ecological factors such as the duration of the reproductive season, the operational sex ratio, the female or male distribution and/or the travel costs to the mate (Riechert 1974, 1981; Fromhage et al. 2007, 2008), unsynchronized male and female maturation may substantially constrain an individual's copulation frequency.

Clade-wide comparisons in mating behavior are essential for revealing macroevolutionary patterns of mating strategies; however, some groups remain largely understudied. Here, we investigate a spider clade informally named "Zygiellidae", which contains temperate and subtropical representatives of several genera exhibiting a moderate sexual-size dimorphism, but diverse entelegyne genital morphologies (M. Gregorič unpublished data). Our ongoing phylogenetic work suggests a close association of the "Zygiellidae" group with the families Nephilidae and Araneidae. Within the former, sexual biology has been well studied in many genera. Many exhibit extreme sexual-size dimorphism and sexual cannibalism, where large females devour tiny males (Kralj-Fišer et al. 2011). In addition, males often engage in genital plugging, genital damage and mate guarding (Schneider et al. 2008; Kuntner et al. 2009a,b). In Araneidae, the sexual biology of most genera remains unstudied, but with some notable exceptions, e.g., *Argiope* (Audouin 1826) with similar sexual phenomena as

Figure 1.—Female (A) and male (B) of the monomorphic *Leviellus thorelli*. Scale bar = 5 mm.

found in Nephilidae (Fromhage et al. 2003; Foellmer & Fairbairn 2004; Zimmer et al. 2012).

To investigate differences and similarities among the three groups, we studied the sexual biology of a previously unstudied "zygiellid" *Leviellus thorelli* (Ausserer 1871) (Fig. 1). To determine whether the *L. thorelli* mating system is monogamous or polygamous, we collected female and male *L. thorelli* and tested them in staged mating experiments. We measured spider body size to estimate the levels of sexual size dimorphism (SSD), observed male-male competition and determined the occurrence of plugging, genital damage and sexual cannibalism.

## METHODS

**Study animals.**—*Leviellus thorelli* spiders were collected in September and October 2009 on houses near Lukovica, central Slovenia (46°09′43″N, 14°41′30″E). We collected 64 adult spiders (33 females and 31 males) and kept them in the laboratory for behavioral trials. We placed the collected females into glass frames to allow them to build webs, whereas males were kept in foam-covered plastic vials. We watered and fed the spiders twice a week with *Drosophila* flies and mealworms and maintained a seasonal light-dark cycle (16:8).

**Experimental protocol.**—In staged mating experiments in the laboratory, we observed mating behavior and occurrences of remating with the same genital organ. Mating was staged by placing a male in the female web, approximately 10 cm away from her. We observed male and female pre-copulatory behavior (courtship), which palp (left/right/both) the male inserted, how long and how many times the male inserted each palp, which female copulatory opening (CO: left/right/both) he inserted into, whether the spiders were aggressive and how they behaved after copulation (e.g., mate guarding). Each observation lasted for two hours. After a trial, we gave a spider 1–12 days of rest before testing for remating.

To make inferences about the mating system, we conducted four types of experimental trials, depending on female and male mating history in the laboratory. We never staged a mating trial between a male and a female that had been previously tested together. In these trials we mated 1) both sexes with unknown mating history [$n$ = 45 trials, $n$ = 64 spiders (28 individuals that did not mate in their first trial were reused)], 2) previously copulated female and male with unknown mating history (female remating, $n$ = 10 trials), 3) female with unknown mating history and a previously copulated male (male remating, $n$ = 8 trials), and 4) both male and female previously copulated [female and male remating, $n$ = 8 trials (2 males used in Experiment 3 were reused)]. When pairing already mated individuals, we devised pairs in such a way that the male could insert his virgin palp only into the female's used CO (insertions were always ipsilateral). For example, we paired a male with a virgin left palp and a used right palp with a female with a used left CO and a virgin right CO; hence, the virgin palp could be inserted only in the used CO and vice versa. If remating did not occur in two subsequent trials, we concluded that remating with the used genital organ was not possible.

In three trials we placed two males on a female's web to document male-male antagonistic behavior. At the end of all trials, the spiders were euthanized, fixed in 70% ethanol and examined morphologically. Voucher specimens are available from the authors.

**Morphological examination.**—We examined all specimens from mating trials for genital damage ($n$ = 64) and measured their first tibia+patella lengths, carapace width and carapace length ($n$ = 50) under a Leica MZ16 stereomicroscope. Following Kuntner & Coddington (2009), sexual-size dimorphism (SSD) is measured as the ratio of female to male body length (or any other size measure).

We macerated all palps in concentrated KOH overnight in order to make them transparent and expandable in distilled water. We excised and examined all epigyna externally, then macerated each epigynal preparation in concentrated KOH overnight, and carefully cleaned it with needles in distilled water (e.g., Kuntner et al. 2009b). This technique exposes the dorsal epigynal anatomy and renders spermathecae translucent, which allows any embolic leftovers lodged inside spermathecae to be seen under a stereomicroscope.

**Statistical analyses.**—We examined the difference in body size measures between the sexes using the Mann-Whitney U Test. Correlations between size measures were analyzed using the Pearson correlation. We used a Generalized Linear Mixed Model (GLMM) to test the effect of two fixed factors, male and female mating history in the laboratory (previously unmated in the laboratory, previously mated in the laboratory) and carapace length; and a random factor (individual code) on occurrence of copulation (yes, no). We sequentially deleted fixed terms in order of decreasing significance; only terms with $P \leq 0.1$ remained in the final model. We re-entered the excluded terms one by one into the final model to confirm that they did not explain a significant part of the variation. We ran all analyses in PASW Statistics 18 (Field 2005).

## RESULTS

**SSD.**—Patella + tibia I, carapace width and carapace lengths were significantly correlated (patella + tibia I, carapace width: $r = 0.63$, $n = 50$, $P < 0.001$; patella + tibia I, carapace length: $r = 0.62$, $n = 50$, $P < 0.001$; carapace width, carapace length $r = 0.71$, $n = 50$, $P < 0.001$). The sexes differed significantly in patella + tibia I length (Mann-Whitney U = 91, $P < 0.001$, $n = 50$) but not in carapace length and width (length: Mann-Whitney U = 254.5, $P = 0.264$, $n = 50$; width: Mann-Whitney U = 231.5, $P = 0.118$, $n = 50$). Using carapace length, SSD in *L. thorelli* was 1.29, which translates to a sexually-size monomorphic species (Kuntner & Coddington 2009).

**Mating results.**—In all staged mating experiments ($n = 71$), a male signaled a female by pulling or drumming on her web. Typically, he initially remained at the edge of the female's web where he attached silk, created a mating thread, plucked the threads of the female's web with his front legs and rubbed his palps. Eventually he walked on the mating thread toward the female resting in her retreat and sometimes touched her legs with his front legs. Then he retreated and rhythmically plucked and beat the mating thread with his front legs. The male repeated this sequence until the female emerged from her retreat, if receptive. During courtship, the female usually moved her first legs and palps and sometimes her abdomen, and turned toward the male. When (if) the female joined the male, they touched with legs in venter to venter position, then suddenly grasped each other with legs to form a ball-shaped outline (S1.—available online at http://www.bioone.org/doi/suppl/10.1636/Hi13-08). The male inserted one of his palps ipsilaterally. After approximately 7 min (mean ± SE, 6.82 ± 1.35 min, $n = 17$) the female and the male abruptly separated, the male usually hanging on the mating thread, and the female retreating (S1). Then, the female typically rubbed her copulatory openings with the third and fourth legs, whereas the male positioned himself approximately 3–5 cm away from the female, plucked the threads, and rubbed and cleaned his palps. A male always continued to court after copulation, but in no case did the pair copulate again. In most trials, the female was not highly aggressive toward the male during or after copulation, and sexual cannibalism was only observed after one mating (5.9%). In some cases, however, the female and the male were aggressive to each other before the copulation, shaking the web and approaching each other with open chelicerae. In such cases, mating never ensued.

If two males were introduced into the same female web, they assumed an aggressive pose toward each other with front legs extended, shook the web, fought vigorously and chased and bit each other. In all three cases the larger male chased off the smaller one (S2.—available online at http://www.bioone.org/doi/suppl/10.1636/Hi13-08).

Of 71 mating trials (Fig. 2), copulation occurred in only 17 cases (23.9%). The occurrence of mating depended on male and female mating history ($F_{95.7,1} = 41.81$; $P < 0.001$). The male and the female copulated in 37.8% ($n = 45$) of the trials when both of them had not previously copulated in our experiments; however, we never observed spiders to copulate in experiments 2, 3 and 4. That is, spiders never remated and reused the genital organ they had previously used ($n = 26$ trials). The random effect was not significant.

**Genital damage.**—Two mated males ($n = 17$) emasculated one palp to become half-eunuchs (Kuntner et al. 2009b) after separating from the females they had copulated with. We found the damaged palps in the males' vials, implying that they were not stuck in the female genitalia during copulation but were rather self-removed after mating. Our morphological examination revealed no further damage to male pedipalps ($n = 31$) or any plug formation in female copulatory openings, ducts or spermathecae ($n = 33$).

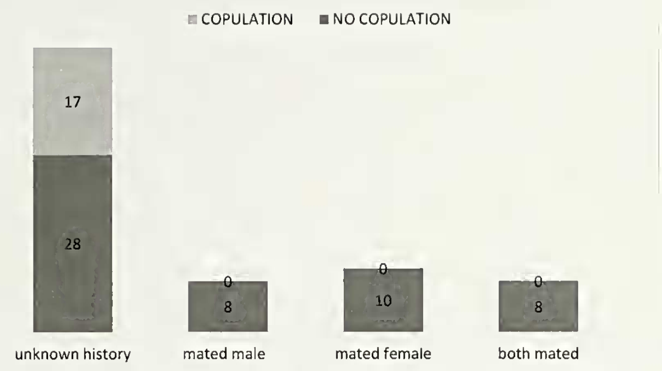

Figure 2.—Copulation success in four different combinations of female and male mating history in the laboratory. Unknown mating history = previously not mated in the lab.

## DISCUSSION

One of our goals was to look for common themes in sexual behaviors of the sexual-size monomorphic "zygiellids" with their close relatives, araneids and nephilids. Copulation behavior of *Leviellus thorelli* resembles that of typical araneid species; males construct and court on a mating thread, with responsive females emerging out of the retreat and copulating with the male in a "hug posture" on the mating thread (Robinson 1982). Similar to other spiders with low levels of SSD, male *L. thorelli* apparently do not damage their genitals obligatorily and do not produce mating plugs, and females exhibit low levels of sexual cannibalism. We found little resemblance to nephilids, where extremely sexual-size dimorphic spiders engage in many ritualistic, sexually conflicted behaviors and strategies (Kuntner 2005, 2006, 2007; Schneider et al. 2005, 2008; Fromhage et al. 2007; Kuntner et al. 2009a, b; Zhang et al. 2011). However, laboratory and field observations of *L. thorelli* indicate intense mate guarding probably due to first-male sperm priority, where males should have reduced fitness benefits when mating with a previously mated female (Austad 1984). Yet, the question of the mating system in *L. thorelli*— and hence questions about macroevolutionary patterns in mating strategies among the three clades—remains open.

Among our aims was to determine the mating system in *L. thorelli*. A male or a female that had previously copulated in the laboratory was never observed remating, which could suggest that both sexes in *L. thorelli* might be either monogamous or at most bigamous. However, we acknowledge here a serious limitation of our study, precluding such definitive conclusion; we collected adult spiders from their natural setting with unknown mating histories, whereas to be conclusive, a study would better rear subadults to ensure virginity. Despite these limitations, the fact is that we never observed polygamy in *L. thorelli*, even though each individual was tested at least twice, with two different potential mates.

Hence, (extreme) polygamy seems unlikely in the system studied.

It is important to note that 60% of pairs failed to mate in the staged experiments. We presume that those spiders had mated before capture. If so, the females that received and stored enough sperm might bias their energy investment in egg production and fertilization, and hence might be sexually unreceptive. It is also likely that females were only receptive during molting and a short period thereafter (e.g., Alcock & Buchmann 1985; Gaskett 2007). Mated or older spider females can be aggressive and exhibit decreased receptivity to subsequent courting males (Elgar 1998), e.g., *Pholcus phalangioides* (Fuesslin 1775) (Schäfer & Uhl 2002), *Argiope keyserlingi* Karsch 1878 (Herberstein et al. 2002) and *Tegenaria atrica* C.L. Koch 1843 (Trabalon et al. 1997).

The alternative/additional explanation for the absence of remating is that males do not find the mated females sexually attractive, as is the case in *Tegenaria atrica* (Trabalon et al. 1997) and *Agelenopsis aperta* (Gertsch 1934) (Papke et al. 2001), both monogamous species that do not produce mating plugs. Male spiders in general prefer virgin over mated females, when females mate only once in several spiders; e.g., *Agelenopsis aperta* (Riechert & Singer 1995). It may vary among species whether a mated male or a female itself reduces female attractiveness. One or more such mechanisms might exist in *L. thorelli*, but this remains to be tested.

Based on our data, we cannot clarify why males did not remate (with the used palp) with a newly introduced female. Research on the closely related *Zygiella x-notata* indicates male choosiness for mates (Bel-Venner et al. 2008; Venner et al. 2010), where only 3% of guarding males switched to another female (Bel-Venner & Venner 2006). Although prolonged tandems during the reproductive season are known to reduce sperm competition and to lower sexual harassment of a mated female (Greenfield & Coffelt 1983; Schöfl & Taborsky 2002), it would be worth studying if and what mechanisms cause *L. thorelli* pairs to persist together in nature, or even to remain monogamous after separation. A phenomenon of prolonged tandems may relate to why no *L. thorelli* males use both palps during mating. In the field and laboratory, we observed that the male persists with the female for a long period with recurrent courting phases. Hence, it is possible that males use both palps with the same female, but over a longer episode than the observed two-hour trial in the laboratory.

Our results show no evidence for genital plugging, but we recorded two cases of male *L. thorelli* becoming eunuchs by severing their palps subsequent to mating. This resembles the eunuch behavior of *Herennia* Thorell 1877 (Kuntner 2005; Kuntner et al. 2009b), but not that of other nephilids where males leave a palp in the female genital tract (Kuntner et al. 2009c; Kralj-Fišer et al. 2011; Li et al. 2012), nor that of *Tidarren* Chamberlin & Ivie 1934 where the single-palped male spontaneously dies while copulating and thus functions as a whole-body mating plug (Knoflach & van Harten 2001). Although the eunuch's behavior in *Leviellus* is clearly not obligate, it may nevertheless be suggestive of some level of post-mating sterility in males.

In conclusion, *L. thorelli* sexual biology resembles that of araneids with low SSD and not that of nephilids, which exhibit pronounced SSD. Although our data require further corroboration with lab-reared spiders, they suggest that the mating system of *L. thorelli* spiders is shaped by a short period of female sexual attractiveness and/or reduced receptivity after mating and intensive mate guarding.

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